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Thanks!

## Methods for Determining "Reference Changes" from Serial Measurements: Plasma Lipid-Bound Sialic Acid

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Lipid-bound sialic (neuraminic) acid (LSA) was measured in EDTA-treated plasma of 26 healthy subjects at three-month intervals for up to one year. The change in LSA concentration for consecutive measurements ranged from -54 to 42 mg/L (mean, -2.1 mg/L; SD, 19.6 mg/L;  $n = 56$ ). The "reference change" for plasma LSA ( $\pm 2$  SD), calculated from distribution of the differences, was  $\pm 39$  mg/L. The 88th percentile of the intra-individual variance was  $338 \text{ mg}^2/\text{L}^2$  and the mean variance was  $159 \text{ mg}^2/\text{L}^2$ . Using the homeostatic, autoregressive time-series model, a reference change of  $\pm 51$  mg/L between two consecutive measurements was determined to be statistically significant (i.e., expected by chance no more than 5% of the time) in 88% of the healthy subjects. Only 73% of the healthy subjects would have had intra-individual variances corresponding to the reference change of  $\pm 39$  mg/L according to the autoregressive model. The concentration of LSA in plasma was significantly decreased upon surgery in five of 10 patients with colorectal adenocarcinomas of Dukes stages A-C when we used  $\pm 39$  mg/L as the reference change, but in only two of the 10 when we used  $\pm 51$  mg/L as the reference change.

**Additional Keyphrases:** statistically significant difference in intra-individual serial data • difference between successive measurements • autoregressive time-series model • intra-individual variance • correlation between successive measurements • colorectal adenocarcinoma • cancer "markers" • neuraminic acid

Recent reports show that various criteria are used to assess the clinical significance of change between serial measurements for a given analyte (1-3). The smallest of the critical changes proposed is often no greater than the analytical error (1, 2). When normal biological variability of the analyte is additionally considered, a true zero difference could be misinterpreted as a clinically significant change.

One commonly used, objective method for assessing the intra-individual variability in concentration of a certain analyte is to define distribution of the observed differences between successive measurements. The critical difference or the "reference change" is then defined as  $\pm 2$  SD of distribution of the differences. However, this reference change does not allow for consideration of variation in the intra-individual variances (4). Autoregressive time-series models permit derivation of other objective criteria to assess the change between two successive measurements (4-6).

In this report, we discuss reference changes for plasma

lipid-bound sialic (neuraminic) acid (LSA), a cancer marker with broad specificity (7-9). Using 26 healthy subjects whose plasma LSA values were determined every three months on two to five occasions, we compared the reference changes calculated from SD of distribution of the differences with the reference change obtained from the autoregressive time-series model with zero correlation between successive measurements. To be consistent with other investigators (4), we used the 88th percentile (closest to the 90th percentile) of distribution of observed intra-individual variances for calculation of the reference change. Based on our clinical experience of the clinical usefulness of plasma LSA, we assessed changes in LSA values of 10 colorectal adenocarcinoma patients upon curative surgery, using these reference changes.

### Materials and Methods

**Specimens and subjects.** Plasma was prepared from blood sampled every three months ( $\pm$  two weeks), with EDTA as the anticoagulant.

The 26 healthy subjects, who were determined to be free from any major illness by medical examination and by review of their medical histories, consisted of 14 men (age range, 42-67 years; mean age, 57 years) and 13 women (age range, 36-59 years; mean age, 45 years). For 16 of these subjects we had at least three serial LSA values. The number of serial measurements for each subject ranged from two to five, with a mean of 3.1.

Plasma LSA values were also obtained for 10 patients with colorectal adenocarcinomas (three with Dukes stage A, five with Dukes stage B, and two with Dukes stage C). Blood specimens were obtained at 0.0-0.3 month (mean, 0.1 month) before curative surgery and three months thereafter. The cancer patients consisted of eight men (age range, 48-69 years; mean age, 61 years) and two women (ages, 60 and 62 years). This group of patients served as a positive control to assess clinical efficacy of the two reference change methods.

**Methods.** Plasma LSA was assayed by the resorcinol colorimetric method, in which sialic acid reacts to form a blue compound, which is measured photometrically at 580 nm (10). Coded plasma samples were shipped on solid  $\text{CO}_2$  to Dianon Systems, Inc., Stratford, CT 06497, and were assayed without knowledge of the subjects' conditions.

**Statistics.** The reference change for plasma LSA was determined from the SD of distribution of the observed differences between successive measurements (5). Alternatively, we used an autoregressive time-series model ( $P = 0.05$ ) with zero correlation between successive measurements. The reference change corresponded to the 88th percentile of distribution of the intra-individual variances. This meant that the calculated change was expected to be

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observed by chance no more than 5% of the time in 88% of the healthy subjects. The autoregressive time-series model with zero correlation between successive measurements (the so-called homeostatic or "white noise" model) is based on the assumption that the values observed in a subject over time vary at random relative to a mean value, and they are characteristic for each subject. When the subject is in control (i.e., clinically stable), the mean and variance are both stable. This homeostatic model is applicable to most analytes of interest in clinical chemistry (6).

## Results

**Distribution of differences between consecutive measurements.** The observed differences between consecutive measurements ranged from -54 to 42 mg/L (mean, -2.1 mg/L; SD, 19.6 mg/L;  $n = 56$ ). Four observed differences between consecutive measurements (-54, -39, -40, and 42 mg/L) exceeded  $\pm 2$  SD of this distribution. The observed differences between consecutive, non-overlapping measurements (5) ranged from -48 to 42 mg/L (mean, -0.9 mg/L; SD, 19.3 mg/L;  $n = 35$ ). The serial plasma LSA values of the 26 healthy subjects are illustrated in Figure 1. The distribution of all the differences is shown in Figure 2A.

**Distribution of intra-individual variances for plasma concentration of LSA.** The observed intra-individual variance for plasma concentration of LSA ( $s^2$ ) ranged from 0 to 684.5  $\text{mg}^2/\text{L}^2$  ( $n = 26$ ). The 88th percentile of this distribution was 338  $\text{mg}^2/\text{L}^2$  and the mean variance was 159  $\text{mg}^2/\text{L}^2$ . Figure 2B illustrates the distribution of the intra-individual variances for plasma LSA. The variance of  $s^2$  was  $2.93 \times 10^4 \text{ mg}^4/\text{L}^4$ . The variance of true intra-individual variances ( $\sigma^2$ ), estimated from the mean and variance of observed variances ( $s^2$ ), was  $1.94 \times 10^4 \text{ mg}^4/\text{L}^4$  (see Appendix). Thus, the variance of  $\sigma^2$  accounted for 66% of the variance of  $s^2$  ( $1.94/2.93$ ). The remaining 34% is due to statistical sampling variability, which would exist even if the true intra-individual variances were constant for all subjects.

**Calculation of reference changes.** Using a homeostatic, autoregressive time-series model (see *Materials and Methods*), we obtained a reference change of  $\pm 51 \text{ mg/L}$  [ $\pm 1.96 \times (2 \times 338)^{1/2} \text{ mg/L}$ ] (5). This reference change corresponded to the 88th percentile of distribution of the observed intra-individual variances and  $P = 0.05$ . That is, a change of  $\pm 51 \text{ mg/L}$  is expected to occur by chance no more than 5% of the time in 88% of the healthy subjects. The greatest difference

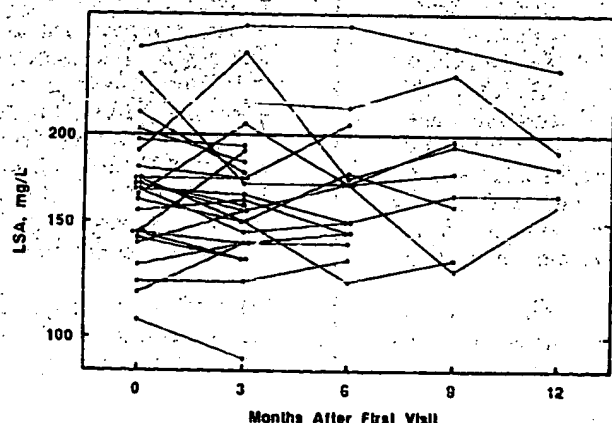


Fig. 1. Serial concentrations of plasma LSA in 26 healthy subjects. The upper reference limit for plasma concentration of LSA (200 mg/L) is represented by the horizontal line.

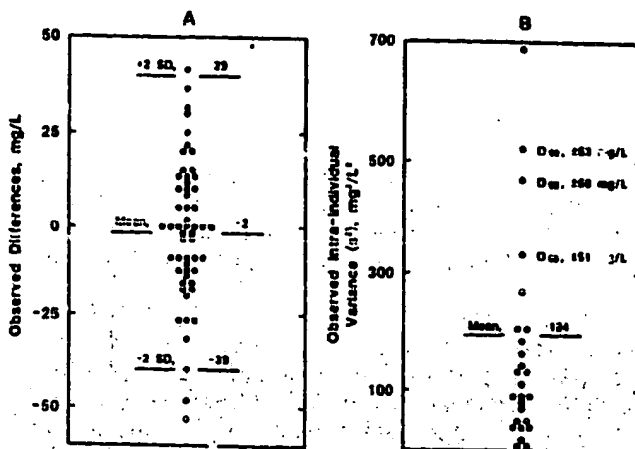


Fig. 2. Distribution of observed differences between consecutive LSA values (A) and that of observed intra-individual variances for plasma LSA (B).

$D_{88}$ ,  $D_{92}$ , and  $D_{98}$  are the reference changes respectively corresponding to the 88th, 92nd, and 98th percentiles of the distribution of  $s^2$ .

between consecutive LSA values was -54 mg/L. This was the only difference that was beyond the reference change of  $\pm 51 \text{ mg/L}$  obtained by the autoregressive model. The reference change of  $\pm 39 \text{ mg/L}$  ( $\pm 2$  SD of distribution of the differences) corresponded to  $s^2$  of 199  $\text{mg}^2/\text{L}^2$ , which was the 73rd percentile of distribution of  $s^2$ . This means that, according to the autoregressive model, a change of  $\pm 39 \text{ mg/L}$  is expected by chance no more than 5% of the time in only 73% of the healthy subjects.

**Effect of curative surgery on the concentration of LSA in plasma of patients with colorectal adenocarcinoma.** The concentration of LSA in plasma changed from -157 to 12 mg/L (mean, -40 mg/L) in the 10 patients with colorectal adenocarcinoma of Dukes stages A-C (see *Materials and Methods*). Five of these patients demonstrated plasma LSA changes of -41 to -157 mg/L (mean, -69 mg/L), which exceeded the reference change of  $\pm 39 \text{ mg/L}$  as obtained from distribution of the observed differences in healthy subjects. However, only two of the 10 patients had changes in their plasma LSA concentrations that were beyond the reference change of  $\pm 51 \text{ mg/L}$  as derived by using the homeostatic, autoregressive time-series model in the 26 healthy subjects. Figure 3 illustrates the effect of surgery on plasma LSA values for the cancer patients.

## Discussion

In this report, we have defined the distribution of the differences between consecutive measurements and the distribution of the observed intra-individual variances for the concentration of LSA in plasma. The variance of true intra-individual variances (5) accounted for most (66%) of the observed intra-individual variances. The reference change obtained from distribution of the differences ( $\pm 39 \text{ mg/L}$ ) was less than the reference change calculated by using the homeostatic, autoregressive time-series model ( $\pm 51 \text{ mg/L}$  for 88th percentile of distribution of  $s^2$  and  $P = 0.05$ ). This is because the SD of the distribution of the differences is not affected by intra-individual variances, but only by the mean intra-individual variances (5). The reference change of  $\pm 39 \text{ mg/L}$  that we obtained from the distribution of the observed differences between consecutive measurements corresponded to only the 73rd percentile of the distribution of the observed intra-individual

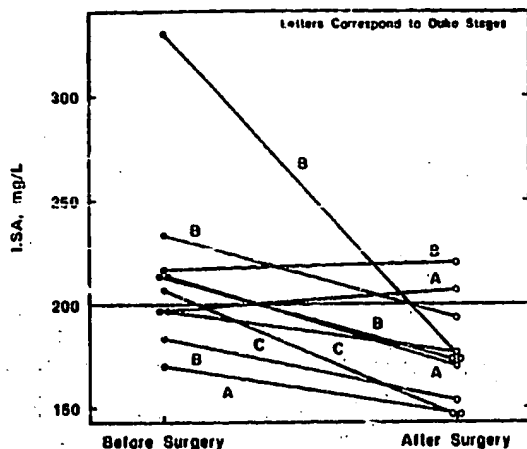


Fig. 3. Plasma concentrations of LSA before and after curative surgery in 10 patients with colorectal adenocarcinomas. The upper reference limit for plasma concentration of LSA (200 mg/L) is represented by the horizontal line. The letters designate Duke's classification for the stage of the disease.

variances. The 52nd percentile of distribution of  $s^2$  corresponded to a reference change of  $\pm 60$  mg/L. The 96th percentile of distribution of  $s^2$  corresponded to a reference change of  $\pm 63$  mg/L, which would flag only one of the 10 LSA changes in the cancer patients as "abnormal." Similar observations of disagreement between these methods for calculation of reference changes have been made by Harris and Yasaka (5) and by Boyd and Harris (4). The reference change obtained from distribution of the observed differences between successive measurements of serum calcium was  $\pm 0.20$  mmol/L (5). This corresponded to only the 35th percentile of distribution of the intra-individual variances. The 90th percentile of distribution of variances corresponded to a reference change of  $\pm 0.24$  mmol/L. The reference change obtained from distribution of the observed differences between successive, non-overlapping measurements of blood urea nitrogen was  $\pm 50$  mg/L (4). This corresponded to only the 48th percentile of distribution of the intra-individual variances. The 90th percentile of distribution of variances corresponded to a reference change of  $\pm 111$  mg/L.

In our study, only one of the 56 (2%) observed differences between consecutive LSA measurements in the healthy subjects exceeded the reference change of  $\pm 51$  mg/L, which corresponded to the 88th percentile of distribution of  $s^2$ . However, four observed differences (7%) exceeded  $\pm 2$  SD of distribution of differences between successive measurements ( $\pm 39$  mg/L). Therefore, the reference change derived by the autoregressive time-series model is more conservative than the reference change obtained from distribution of observed differences. This also seems to be the case when one considers that only two cancer patients were found to undergo significant decreases of their plasma LSA concentrations upon surgery (using either the 88th percentile or the 92nd percentile of distribution of  $s^2$ ), whereas five patients showed significant decreases in their plasma LSA values upon surgery as indicated by the reference change obtained from distribution of the differences. Based on our

observations in support of the clinical usefulness of plasma LSA assay in patients with colorectal adenocarcinomas as well as in patients with neoplastic colorectal polyps, we recommend use of  $\pm 39$  mg/L (obtained from distribution of observed differences) as the reference change for plasma concentration of LSA. Although 7% of the observed changes in the healthy subjects were beyond this reference change, five of 10 decreases (50%) in plasma LSA values measured after curative surgery were found to be significant. The homeostatic, autoregressive time-series model may be more useful when the baseline values of each patient are used to identify "abnormal" changes, such as in monitoring recurrence of cancer in patients who are in remission (6). We are currently assessing these and other methods for determination of reference changes for other serological cancer markers. Furthermore, we are applying these findings to the monitoring of cancer patients who are in remission, to identify clinically significant increases associated with recurrence of the disease.

## Appendix

The variance of true intra-individual variances ( $\sigma^2$ ) can be estimated from the mean and variance of observed intra-individual variances ( $s^2$ ) by the following formula:

$$\text{Var } \sigma^2 = [\text{Var } s^2 - (2/n - 1)(\text{mean } s^2)^2](n - 1)/(n + 1)$$

where  $n$  is the mean number of intra-individual measurements.

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